J. Am. Chem. Soc. 1994, 116, 7955–7956

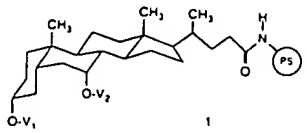
Peptidosteroidal Receptors for Opioid Peptides. Sequence-Selective Binding Using a Synthetic Receptor Library

Rustum Boyce, Ge Li, H. Peter Nestler, Toshiro Suenaga, and W. Clark Still*

Department of Chemistry, Columbia University New York, New York 10027

Received May 9, 1994

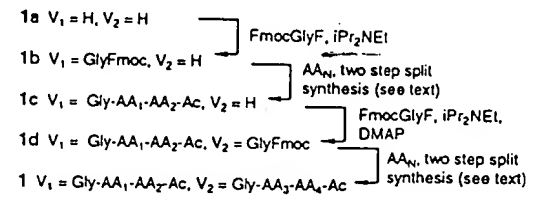
For several years now, workers in the field of molecular recognition have labored to create synthetic receptors that selectively bind simple organic or biological substrates. While there have been notable successes,[1] these have typically involved construction of cagelike host structures for small substrates such as simple heterocycles or single biopolymer residues. Though natural receptors (*e.g.*, antibodies) can bind much larger oligomeric substrates with high selectivity, no one has yet described a workable approach to synthetic receptors having analogous properties. In this communication, we report the first example of a practical method for creating new receptor molecules which, in principle, may have any desired substrate specificity. Our approach is taken from nature, which often solves binding problems by screening a large, diverse set of related receptors. In the example we report here, our receptors are simple peptidosteriods (**1**) which consist of A,B-*cis*-steroidal cores and peptidic appendages (V_n) which we synthesize in 10^4 different forms by encoded combinatorial chemistry.[2] As we will show using a series of opioid peptides as substrates, different substrates preferentially bind different members of our peptidosteroidal receptor library.



1

The scheme we use entails creating a library of diverse receptors and then selecting particular receptors which bind some desired substrate.[3] For our receptor library, we chose a very simple design which is diagrammed as **1**, where V_1 and V_2 represent various tripeptide appendages and the circle represents a polystyrene (PS) bead. Though peptidosteroid **1** bears little resemblance to a typical host, which is often a conformationally restricted macrocycle, it does carry an assortment of hydrogen bond donors and acceptors for binding peptidic substrates in organic solvents and is readily assembled by simple chemistry in high diversity. The library's only preorganization comes from the rigid nucleus of cholic acid, which serves as a scaffolding that carries functionality for appending diversity and connecting to the solid support.[4] The question we ask here is whether or not such a simple receptor library includes molecules which will associate selectively with certain, arbitrarily chosen tetra- or pentapeptides. Below we outline the synthesis of receptor library **1**, the assay we use to screen for substrate binding, and the binding properties of selected receptors using various enkephalin-like peptides as substrates.

Our library synthesis (below) began with cheno(12-deoxy)-cholic acid which we attached to 50–80 μm (aminomethyl)-polystyrene beads using diisopropylcarbodiimide to give **1a**. We then acylated the less hindered C3 hydroxyl using 1.5 equiv of Fmoc-glycyl fluoride in DMF (triple coupling) to give **1b** with <5% reaction at C7.[5] Next, we used two rounds of split synthesis[6] to append all possible dipeptides made from the 10 Fmoc-(L)-amino acids Ala, Val, Leu, Phe, Pro, Ser(tBu), Thr(tBu), Lys-(Boc), Asp(tBu), and Glu(tBu). Thus we added AA_1 and AA_2 to the C3 glycine and then capped the V_1 chain with AcOH to produce **1c**. This combinatorial synthesis led to 10^2 variants of V_1 and was encoded with eight molecular tags using the binary tagging method described previously.[2a] To glycylate the hindered C7 hydroxyl yielding **1d**, we again used Fmoc-glycyl fluoride but employed 4-(*N*,*N*-dimethylamino)pyridine to catalyze the coupling.[5] Finally, the encoded split synthesis procedure above was again employed with eight more tags to complete V_2 by adding AA_3, AA_4, and Ac. This double split synthesis led to a 10^4-member library of peptidosteroids **1** in which each different member of the library was attached to a different synthesis bead.



To test our receptor library for receptor–substrate binding and sequence selectivity, we chose four closely related opioid peptides (enkephalins) as substrates. The binding screen was conducted as a solid phase assay in which a sample of the initially colorless bead-bound receptor library (**1**) was treated with a dilute solution of substrate tethered to an intensely colored dye. Binding was detected by simple inspection: receptor library beads which bound substrate picked up the color of the dye. The dye we used was Disperse Red 1 (Aldrich), which we acylated with glutaric anhydride to produce dye-$CO(CH_2)_3COOH$. This dye-linker was then used to N-acylate four enkephalin-like peptides to yield dye-labeled substrates **2**–**5**.

2 dye-$CO(CH_2)_3$CO-Gly-Gly-(L)Phe-(L)Leu

3 dye-$CO(CH_2)_3$CO-(L)Tyr-(D)Ala-Gly-(L)Phe-(L)Leu

4 dye-$CO(CH_2)_3$CO-(L)Tyr-Gly-Gly-(L)Phe-(L)Leu

5 dye-$CO(CH_2)_3$CO-(L)Tyr-Gly-Gly-(L)Phe-(L)Met

Receptor library **1** was then screened for binding with ~100 μM-red Leu enkephalin (**4**) in $CHCl_3$. After agitating the mixture for 48 h to establish equilibrium, we found that many beads had developed light orange colorations but that only a few (~1%) had turned bright red (see Figure 1a). We picked ~50 of these bright red beads and decoded their synthetic histories by gas chromatography to identify AA_1–AA_4 for those receptors which selectively bound **4**.[3] As described below, we found significant preferences for certain amino acids at each of the receptor's four AA_N sites.

(1) Recent reviews: Schneider, H.-J. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 848. Webb, T. H.; Wilcox, C. S. *Chem. Soc. Rev.* **1993**, 383.

(2) (a) Ohlmeyer, M. H. J.; Swanson, R. N.; Dillard, L. W.; Reader, J. C.; Asouline, G.; Kobayashi, R.; Wigler, M.; Still, W. C. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 10922. (b) Brenner, S.; Lerner, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 5381. (c) Kerr, J. M.; Banville, S. C.; Zuckermann, R. N. *J. Am. Chem. Soc.* **1993**, *115*, 2529.

(3) Similar approach using library of substrates: Borchardt, A.; Still, W. C. *J. Am. Chem. Soc.* **1994**, *116*, 373. Yoon, S.-S.; Still, W. C. *Tetrahedron*, in press.

(4) Cholic acids are well-known building blocks for host molecules: Davis, ... *Chem. Soc. Rev.* **1993**, 245 and references therein.

(5) Carpino, L. A.; Mansour, E. M. E.; Sadat-Aalaee, D. *J. Org. Chem.* **1991**, *56*, 2611; 2635. Bertho, J.-N.; Loffet, A.; Pinel, C.; Reuther, F.; Sennyey, G. *Tetrahedron Lett.* **1991**, *32*, 1303.

(6) (a) Furka, A.; Sebestyen, M.; Asgedom, M.; Dibo, G. *Abstr. 14th Int. Congr. Biochem. (Prague, Czechchoslovakia)* **1988**, *5*, 47. (b) Furka, A.; Sebestyen, M.; Asgedom, M.; Dibo, G. *Abstr. 10th Int. Symp. Med. Chem. (Budapest, Hungary)* **1988**, 288. (c) Furka, A.; Sebestyen, M.; Asgedom, M.; Dibo, G. *Int. J. Pept. Protein Res.* **1991**, *37*, 487. (d) Lam, K. S.; Salmon, S. E.; Hersh, E. M.; Hruby, V. J.; Kazmierski, W. M.; Knapp, R. J. *Nature* **1991**, *354*, 82. (see also *Nature* **1991**, *358*, 434.) (e) Zuckermann, R. N.; Kerr, J. M.; Siani, M. A.; Banville, S. C. *Int. J. Pept. Protein Res.* **1992**, *40*, ...

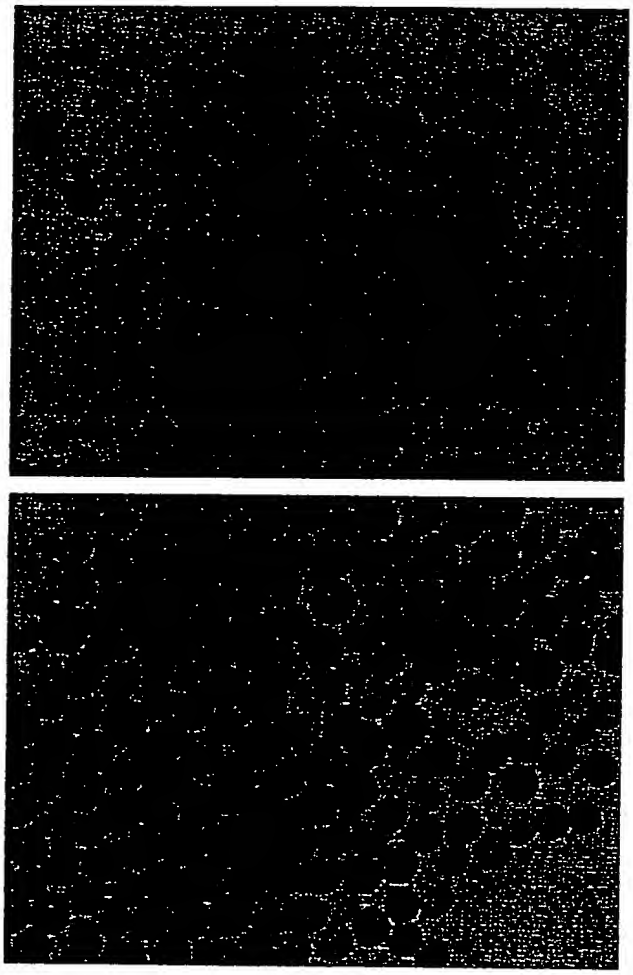


Figure 1. (a, top) Bead-bound receptor library 1 stained by Red-4. (b, bottom) Bead-bound receptor library 1 stained by Red-3 and Blue-4.

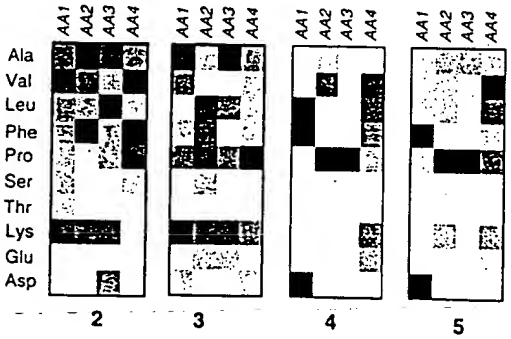


Figure 2. Histograms of AA_1–AA_4 in receptors 1 which bind 2–5.

Similar screenings of the receptor library were carried out using the other red substrates (2, 3, and 5), and the decoding results for the most tightly binding receptors in each case are illustrated as gray-scale histograms in Figure 2. These histograms give the frequencies of various amino acids in the appendages of substrate-binding receptors where the gray scale represents the fraction (white, 0%; black, ≥50%) of each different amino acid at receptor sites AA_1–AA_4 for each substrate.[7] These diagrams show that substrates 4 and 5 are both bound by closely related subsets of the receptor library and that receptors selected by both 4 and 5 have a strong preference for Pro at the AA_2, AA_3 sites. This preference for Pro (60% of all 4- or 5-selected beads at AA_2 and 90% at AA_3) is not found in receptor library members selected by assays using red-2 and red-3 as substrates. In these cases, Pro appeared at AA_2 and AA_3 in <10% of the beads we decoded. As indicated by the gray-scale histograms, substrates 2 and 3 bind receptors with less selectivity for particular residues at particular AA_N sites, but it is clear that the receptor populations selected by 2, 3, and 4/5 all differ from one another. These results establish that different substrates are bound by different populations of our receptor library.

Though our solid phase assay selects those receptors which most tightly bind a given substrate, such receptors are not necessarily the ones which bind that substrate most selectively, *e.g.*, which bind 4 but not 3. To find selective receptors, we developed a related screen which we term a *two-color assay*. The idea is to attach differently colored dyes to substrates to be distinguished and then to screen for library beads that pick up only one color. For example, upon treating our receptor library with a mixture of red-3 and blue-4 (made with Disperse Blue 3[8]), we found a variety of purplish to reddish-purple (nonselective or slightly 3-selective) beads as well as a few very blue (4-selective) beads (see Figure 1b). Upon decoding these blue beads, we found mainly appendage sequences for 1 which were closely related to the consensus sequences found with the single-color red-4 assay including AA_1–AA_4 = PheProProLeu (**1FPPL**) and AspProProVal (**1DPPV**). We also decoded several of the purple beads and found PheAlaProVal (**1FAPV**) and PheLysPhePro (**1FKFP**).

To verify that receptors from the blue beads were actually selective for 4, we resynthesized receptors **1FPPL** and **1DPPV** and used HPLC to measure their relative binding of 3 and 4 (both labeled by the same red dye). We found that both receptors did, indeed, bind red-4 more tightly than red-3, and the selectivity (ΔG_{4-3}) was −1.0 and −1.6 kcal/mol with **1FPPL** and **1DPPV**, respectively. In contrast, resynthesized **1FAPV** and **1FKFP** from the purple beads bound red-3 and red-4 without measurable selectivity. Though **1FPPL** and **1DPPV** are thus able to distinguish two pentapeptides which differ by a single amino acid, our simple receptor library 1 does not seem to contain members capable of making some other distinctions. For example, we were unsuccessful in finding receptors which clearly distinguished labeled Leu[5] enkephalin (blue-4) and labeled Met[5] enkephalin (red-5). Indeed, between 4 and 5, the most 4-selective receptor we could find was **1DGVG** (ΔG_{4-5} = −0.1 kcal/mol), and the most 5-selective receptor was **1LKAP** (ΔG_{5-4} = −0.2 kcal/mol).

Up to now, synthetic receptors have been individually tailored to fit desired substrates using preorganization as the guiding principle. In the work described here, we demonstrate the power of an alternative approach which relies more on diversity generation and screening than on rational design. Given that peptidosteroids 1 bear little resemblance to typical host molecules, it is remarkable that our rather small library contains receptor-like structures which bind and distinguish oligopeptides with selectivities which would be difficult to engineer by traditional deterministic means. Though the extent of sequence-selective binding is limited with library 1, it is likely that we can do much better. Thus we expect that significant improvements can be gained with libraries incorporating better-defined substrate binding sites, reduced structural flexibility, and increased diversity. In any event, this work demonstrates that deterministic design is not the only approach to new molecules having desirable properties. Indeed, the best approach would appear to result from combining a good *design* with efficient methods for *diversity generation and screening*.

Acknowledgment. We acknowledge National Science Foundation Grant CHE92 08254 (W.C.S.) and Liebig Fellowship from the "Fonds der chemischen Industrie" (H.P.N.).

(7) The exact receptor sequences found depended upon the concentration of substrate used in the assay, because the smallest binding energy detectable varies with concentration. With substrates 2–5, we adjusted substrate concentrations individually to give ~1% deep red staining of the library.